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Supplemental Information

Metabolic Regulation

of Brain Response to Food Cues

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Supplemental Inventory

Table S1, Related to Figure 3. Blood Sample Analyses

	0 cal mean(SD)	**112.5 cal mean(SD)**	**p**
Δ glucose	-4.54(5.88) mg/dL	48.70(14.10)	<0.001
Δ ghrelin	20.34(98.89) pg/mL	-115.05(92.11)	0.010
Δ insulin	-2.42(3.54) μIU/mL	31.65(9.12)	<0.001
Δ triglycerides	3.76(8.18) mg/dL	0.99(5.29)	0.264
Δ hematocrit	0.44(1.24) %	-0.40(0.86)	0.066
Δ hemoglobin	0.11(0.43) g/dL	-0.07(0.34)	0.290

Despite significant effects of caloric load on ghrelin and insulin, these measures do not significantly predict difference brain response to the two flavors paired with the different caloric doses.

Supplemental Experimental Procedures

Subjects

Subjects were 14 right-handed nonsmokers, taking no daily medication and having no known history of loss of consciousness, chemosensory impairment, neurological, or psychiatric disorders. Eight were women. The average age (mean/standard deviation) was 24.64/6.24 and the average body mass index was 22.01/3.1. All gave written informed consent to participate in our study that was approved by Yale University School of Medicine Human Investigation Committee.

Procedure

Subjects participated in a pretest, a series exposure sessions (6 exposures per stimulus), a posttest, and an fMRI scanning session (see Figure 1). All sessions occurred within a 3-week period.

Screening

Screening was performed over the phone to insure that subjects were right-handed nonsmokers between the ages of 18 and 45 with no known history of psychiatric disorders, food allergies, diabetes, deficits of taste and smell, and no contraindications for fMRI (left-handed, metal in body, claustrophobia, closed head injury with loss of consciousness).

Pretest

Subjects arrived at the laboratory following a 4-hour fast (water ok until one hour before the session). Consent was then obtained and subjects filled out the Three Factor Eating Questionnaire (TFEQ), which provides measures of dietary restraint, disinhibition, and hunger [1], as these factors have been shown to influence flavor nutrient conditioning [2, 3]. Average TFEQ score was 5.43±3.25; subjects scoring ≥13 were excluded (approximately one out of every five subjects were excluded based upon this criterion). To increase compliance with fasting, a cheek swab sample was collected at the beginning of all sessions. Subjects were told that this sample would "be tested for markers of internal state" that would indicate whether they were sufficiently fasted. In actuality, these swabs were discarded.

Scale Training: Next subjects received training on the use of the rating scales. Internal state (hunger, fullness, and thirst) was rated using a visual analogue scale (VAS) with the left anchor "Not Hungry/Full/Thirsty at All" and the right anchor "Very Hungry/Full/Thirsty"). Wanting was rated on a VAS (Wanting: left anchor = I do not want to drink this and right anchor = I want to drink this more than anything). Overall stimulus intensity and sweetness intensity were measured with the general labeled magnitude scale (gLMS) [4, 5]. Liking was measured with the labeled hedonic scale (LHS) [6]. Subjects were instructed in scale use and were given practice rating real and imagined stimuli on these scales. At the end of scale training, subjects rated two concentrations of sucrose solution (1.0 M and 0.32 M) on the LHS. If ratings indicated that they disliked either solution they were excluded. This was done because there is evidence that sweet taste liking influences flavor nutrient conditioning [7]. Approximately one out of every five subjects screened were excluded based on this criterion.

Rate Flavors: Next subjects used the scales to rate the flavor stimuli. Flavor stimuli were formulated to be novel, distinguishable, close to neutral in pleasantness and similarly pleasant and intense to each other. Ten beverages were created with these criteria in mind following a series of pilot experiments with commercially available ingredients. All selected flavored beverages contained 0.1% (w/v) citric acid (Sigma-Aldrich), 0.0078% sucralose (Sigma-Aldrich), a flavor (Bell Labs Flavors and Fragrances, Inc., IL, USA), and a unique color (McCormick & Co, Inc. MD, USA). Flavor-color pairings were counterbalanced across subjects, but kept consistent across all sessions within each subject. Critically, during the pretest, posttest and fMRI session all flavors were non-caloric. Subjects were presented with 10-ml of each stimulus in 30-ml plastic medicine cups and instructed to pour the entire content into their mouths, swish it around, expectorate it into the sink and then rate overall intensity, sweetness, liking, and wanting. After providing the ratings the subjects rinsed their mouths with deionized water and then waited 30 seconds before beginning the next trial. Each of the 10 flavored beverages was presented three times in random order. Subjects were only asked to continue in the study if they consistently rated 3 flavors as similarly pleasant, with the criterion that ratings had to fall between neutral and like moderately on the LHS. Fifteen

subjects were excluded due ratings outside the target range (e.g. flavors rated as too pleasant or unpleasant, or lacking two flavors of similar pleasantness).

Determine Beverages: Average ratings for each stimulus were calculated and inspected to determine if there were two stimuli that were close to neutral in liking and equally liked. A second experimenter selected one of the flavors to be paired with 0 calories (0-calorie beverage) and one to be paired with 112.5 calories (112.5-calorie beverage) during the exposure sessions. To maintain a double-blind paradigm this experimenter prepared the beverages for the exposure sessions and did not inform the first experimenter of the calorie-flavor designations. Importantly calories were added to the 112.5-calorie beverage with maltodextrin, a carbohydrate that breaks down into glucose and is generally undetectable in foods and beverages. To verify that study participants were unable to detect the presence of maltodextrin they participated in triangle tests in which they indicated which of three cups was different. All cups contained the same flavor but for each trial either one or two cups also contained maltodextrin. Eight trials were conducted. Subjects continued in the experiment only if the results from the triangle test indicated that they could not reliably detect the presence of maltodextrin. One subject was excluded from the study after being determined able to detect maltodextrin in solution.

fMRI Training: Subjects were then brought to the fMRI simulator and experienced a 13-minute mock fMRI run during which they received liquids as described in Supplementary Figures 3 and 4. The purpose of the mock scan was to train subjects and screen out those who found the procedure uncomfortable. In addition, subjects were presented with four versions of a tasteless/odorless solution (2.5 mM sodium bicarbonate and 25mM potassium chloride, plus three dilutions at 25%, 50%, and 75% of the original concentration) and asked to select the one that tasted most like nothing. This mixture was then used as the control “tasteless” stimulus during fMRI scanning.

Exposure Sessions

Subjects were exposed six times to the 0-calorie beverage and six times to the 112.5-calorie beverage. For each beverage four of these exposures were in the lab and two were at home.

In Lab Lunch Session: Subjects arrived at the lab an hour before their typical lunchtime, having undergone similar instructions to fast as in the pretest. Upon arrival at the lab subjects wrote down what they ate for breakfast. A cheek swab sample was then collected and subjects rated their internal state (hunger, fullness and thirst). Subjects then drank the experimental beverage. A second set of internal state ratings was collected immediately after consumption. Subjects then either read quietly or watched a DVD of a sitcom for 30 minutes. A third set of internal state ratings was collected and then the subject was provided with their selected lunch, which they ate in the laboratory. A final set of internal state ratings was made before the subject left.

In Lab Dinner Session: Four hours following the lunch session subjects returned to the lab for the dinner session. They were asked not to eat or drink anything during the four hours (water ok). The dinner sessions were identical to the lunch session except that after the 30-minute rest period, and third set of internal state ratings, they returned home for their dinner. Before leaving subjects were given an additional beverage and paper rating scales and instructed to drink the beverage and make ratings when they woke the following morning.

Importantly the same beverage is consumed at these three time points (in lab lunch, in lab dinner, and at home breakfast), which are collectively designated as exposure session 1. There are four exposure sessions in total; two for each beverage.

Blood Draws: In order to determine the influence of the beverages upon peripheral physiology blood samples were collected at one of the lunch exposure sessions for each beverage. The IV was inserted after the cheek swab sample was collected. After IV insertion, subjects waited 30 minutes to stabilize before the blood was drawn prior to drink consumption. A second blood sample was taken after the 30-minute wait period and the IV was removed (before lunch). Thus, lunch exposure sessions were 30 minutes longer when bloods were drawn. The blood samples were immediately spun down and glucose, insulin, ghrelin, triglycerides, hematocrit and hemoglobin assayed. A small aliquot of whole blood (~0.3ml) was used for the immediate analyses of hematocrit and hemoglobin. Another aliquot was transferred into

a tube with potassium EDTA anticoagulant for the determination of ghrelin. The remaining sample was transferred into a tube with no anticoagulant for the determination of blood insulin, glucose and triglycerides. The samples were centrifuged, frozen immediately and stored at –80°C until analysis. Serum concentration of insulin and plasma concentration of ghrelin was measured using the competitive binding radioimmunoassay method. Intra and inter assay coefficient of variation for the mid-range standard for insulin [45 (4.5) uIU/ml] was 2.3% and 3.7% (Siemens Healthcare Diagnostics, Los Angeles, CA). Intra and inter assay coefficients of variation for ghrelin (standards low range 418-868 pg/mL) were 1.6% and 2.9% (Millipore Corp., Billerica, MA). Plasma glucose and triglycerides were measured using the modified Trinder method through a colorimetric endpoint (Eagle Diagnostics, DeSoto, TX). Whole blood hemoglobin is also a colorimetric assay based on the determination of cyanmethemoglobin (Eagle Diagnostics, DeSoto, TX). Hematocrit is measured by drawing up whole blood into heparinized hematocrit tubes and centrifuged for 3 minutes. The percentage of red blood cells vs. plasma is read on a micro-capillary plate.

Posttest

Subjects arrived fasted, as in the previous sessions, and a cheek swab sample was collected. Subjects then received scale training and rated the 10 flavors that had been rated in the pretest (including the 0-calorie paired flavor and the 112.5-calorie paired flavor). Critically, as in the pretest, none of the flavors contained calories. Following these ratings subjects completed the Dutch Eating Behavior Questionnaire to further assess eating style[8] and the Nutrition Questionnaire to assess diet [9, 10].

fMRI Session

The fMRI session was conducted on a different day than the posttest. Subjects arrived fasted, as in prior sessions and provided the cheek swab sample. Following pregnancy tests subjects changed into a hospital gown and were inserted into the scanner and fitted with the flavor delivery device. In brief, flavors are contained in 60 ml disposable syringes mounted on programmable syringe pumps (Braintree Scientific, Braintree, MA) and controlled by programs written using Matlab (MathWorks Inc., Sherborn, MA) and Cogent2000 v1.25 (Wellcome Department of Cognitive Neurology, London, UK). Liquids are infused at a rate of 15mL/min. Each syringe is connected to a 25-foot length of Tygon beverage tubing (Saint-Gobain Performance Plastics, Akron, OH) with an inside diameter of 3/32". All tubing terminates into a custom-designed Teflon, fMRI compatible gustatory manifold (designed and constructed in the Pierce Laboratory shop) anchored to the MRI headcoil (wooden replica in the photo). **b,** Photograph of a side view of the gustatory manifold. All flavors and rinses (tasteless/odorless solutions) pass through 1-mm channels that converge at a central point at the bottom of the manifold for delivery to the tongue tip. To prevent the subject's tongue from coming in contact with the 1mm holes, and to ensure the liquids flow directly onto the tongue, a short silicone tube is attached to the outflow point under the hole. The tip of the tongue rests comfortably against the lowest point of the tube. A large vent hole prevents subjects from drawing or sucking the stimulus through the manifold at uncontrolled times or rates. The gustatory manifold is attached to an anchoring block that clamps onto the front of the head coil. The anchor height and horizontal positions are adjustable via two knobs accessible to the subject and the experimenter. The manifold is then locked in place for the duration of the scanning run.

During scanning subjects received the tasteless control liquid as well as three flavors; the 0-calorie paired flavor, the 112.5-calorie paired flavor, and a control flavor, selected from the original set of 10 flavors as being closest in liking to the flavors chosen to be the exposed flavors (0-calorie paired flavor and 112.5-calorie paired flavor). Subjects rated their internal state and each of the 4 stimuli (as in pretest). Each stimulus was delivered 27 times over three 12-min and 24-sec second runs. Images were acquired on a Siemens Trio TIM 3.0 T scanner. Echo planar imaging was used to measure the blood oxygenation-level dependent (BOLD) signal as an indication of cerebral brain activation. A susceptibility-weighted single-shot echo planar method was used to image the regional distribution of the BOLD signal (TR/TE 2000/20ms; flip angle, 80°; FOV, 220 mm; matrix, 64 x 64; slice thickness, 3 mm; number of slices, 40). Slices were acquired in an interleaved mode to reduce the cross talk of the slice selection pulse. At the beginning of each functional run, the MR signal was allowed to equilibrate over six scans ("dummy images") for a total of 12 s, which were then excluded from analysis. The anatomical scan used a T1-weighted 3D FLASH sequence (TR/TE, 2530/3.66 ms; flip angle, 20°; matrix, 256 x 256; 1 mm thick slices; FOV, 256; 176 slices).

Data Analyses

fMRI Data were analyzed on Linux workstations using Matlab (MathWorks, Inc., Sherborn, MA) and SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK). Functional images were slice-time acquisition corrected using sinc interpolation to the slice obtained at 50% of the TR. All functional images were then realigned to the scan immediately preceding the anatomical T1 image. The images (anatomical and functional) were then normalized to the Montreal Neurological Institute template of grey matter, which approximates the anatomical space delineated by Talairach and Tournoux[11]. Images were then detrended, using a method for removing at each voxel any linear component matching the global signal[12]. Functional images were smoothed with a 6 mm FWHM isotropic Gaussian kernel.

For the time-series analysis on all subjects, a high pass filter (300 sec) was included in the filtering matrix (adapted to the period of presentation in this long event-related paradigm) in order to remove low-frequency noise and slow drifts in the signal. Condition-specific effects at each voxel were estimated using the general linear model. The response to events was modeled by a canonical hemodynamic response function (HRF) included in SPM8. The temporal derivative of the hemodynamic response function was also included as part of the basis set to account for up to 1-sec shifts in timing of the events[13]. The events of interest were the four different stimuli ("control", "0-calorie paired flavor", "112.5-calorie paired flavor" and "tasteless"). To capture the neural response associated with flavor specifically, the events were modeled with an onset at the end of delivery when the stimulus is swallowed and retronasal olfactory perception occurs. The events were modeled as mini-blocks with a duration that corresponded to the variable interval until the rinse or the next stimulus rinses were modeled as nuisance effects. No head movement regressors were included, as subjects with head movements beyond 1 mm were excluded from the analysis (3 subjects were excluded from the analyses due to swallowing or other related head movements > 1mm). Comparisons between events of interest were performed at the subject level (112.5-calorie paired flavor vs. 0-calorie paired flavor; 112.5-calorie paired flavor vs. tasteless; 0-calorie paired flavor vs. tasteless; and control flavor vs. tasteless). Parameter estimate contrast images from each subject were then entered into a second level random effects analysis and contrasts of interest were defined. T-maps were thresholded at uncorrected $P=0.005$, and subsequently cluster-level inferences were applied to the resulting images. Cluster activations were considered significant at $p<0.05$ with p-values adjusted according to the false discovery rate (FDR) criteria. Local maxima within clusters were reported as displayed by the SPM8 Results tables. Reassuringly, significant clusters only included regions that have been implicated in flavor-nutrient conditioning by animal work[14-19]. Anatomical locations were confirmed using the WFU Pick Atlas tool in SPM8[20].

We used whole-brain regression analyses in SPM8 to determine whether and where we observed significant correlations in neural response to the flavors and measures of hedonic (change in pleasantness rating from pre to posttest) or metabolic conditioning (change in insulin/ghrelin/glucose pre vs. post drinking the 112.5 calorie beverage vs. 0 calorie beverage)[21, 22]. For each contrast of interest (e.g. 112.5-calorie paired flavor vs. 0-calorie paired flavor), the parameter estimate images from each subject were entered into a regression design along with a vector comprising the hedonic or metabolic variable of interest. For example, the vector to determine the influence of blood glucose on brain response contained a single value for each subject that was calculated by subtracting glucose levels prior to drink consumption from the levels 30 minutes later (Δ), and then by subtracting Δ for the 0 calorie exposure drink from Δ 112.5 calorie drink. For each value in these vectors we used a z-score or standardized score. The z-score was obtained for each subject by first calculating the mean and standard deviation of all scores for that subject on the variable of interest. Subsequently we subtracted the mean from each individual value and then divided the resulting value by the standard deviation. In a separate step we calculate the magnitude of the correlation (r) in the maximally activated voxel (as SPM8 does not give this information) for post hoc illustrative purposes only.

Finally, to further confirm that liking subjective ratings failed to produce significant effects on flavor responses in regions influenced by blood glucose levels, further region-of-interest analyses on glucose-modulated loci were performed to provide additional evidence that glucose levels, but not flavor liking, accounted for the results obtained. Accordingly, the postconditioning ratings were subtracted from each other (i.e. postconditioning CS+ ratings minus postconditioning CS- ratings), and the resulting quantities were regressed to the brain responses obtained from the contrast [CS+ minus CS-]. For each of the glucose-modulated regions (*i.e.* NAcc and hypothalamus), small-volume corrections of p-values associated with local maxima were performed by defining a 6mm-radius sphere centered on these maxima. Statistical p-values were FDR-corrected at the cluster level across the whole brain, *i.e.* the

corrected p-value accounts for the number of expected activation clusters across the entire image (which in this case equals to 16.04 clusters).

In the neuroimaging analyses involving regressions against glucose levels and liking ratings, 14 subjects were included. When hunger ratings were used as regressors, only 13 subjects were entered in the analyses since hunger ratings for one subject were not recorded. The values from this particular subject were also not recorded for the triangle test.

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